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Comparative study of the immunoprotective effect of two grass carp-sourced *Bacillus subtilis* spore-based vaccines against grass carp reovirus

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A B S T R A C T

Grass carp reovirus II (GCRV II) causes severe haemorrhagic disease with high mortality in grass carp, *Cyenopharyngodon idellus*. Vaccination is a very effective method in conferring protection against GCRV. In the current study, we constructed two grass carp-sourced *Bacillus subtilis* spore-based vaccines (GC5-VP4 and GC5-NS38) and investigated their potential as oral candidate vaccines against GCRV II. Our results showed that both VP4 and NS38 proteins of GCRV II were efficiently displayed on the spore surface of *B. subtilis* GC5, and the two recombinant spores stayed in the hindguts of grass carp for antigen presentation. After oral administration, compared with the control groups, both recombinant vaccines GC5-VP4 and GC5-NS38 increased the survival rate of grass carp against GCRV II, with relative percent survival rates of 30% and 36.4%, respectively. Although they had a similar immunoprotective effect, the mechanisms were very different. GC5-VP4 mainly elicited specific antibody responses, both systemic and mucosal, whereas GC5-NS38 mainly elicited an inflammatory response and cellular immunity via expression of major histocompatibility complex molecules. Furthermore, this study demonstrated that the recombinant spores triggered high levels of mucosal immunity and would be promising oral candidate vaccines against GCRV II in grass carp. Combined with previous research, we inferred that vaccine efficacy was not only related to antigen, but also vaccination strategies.

1. Introduction

Global fish production from aquaculture has grown rapidly over the past decades, and grass carp (*Ctenopharyngodon idella*) shares the largest portion (about 17.9%) of.

freshwater production in China. However, haemorrhagic disease caused by grass carp reovirus (GCRV) results in a tremendous loss in this industry. GCRV is a double-stranded RNA virus belonging to the genus *Aquareovirus*, family *Reoviridae* (Fauquet & Stanley, 2005), with three genotypes (GCRV I, GCRV II and GCRV III), among which GCRV II is the most epidemic isolate (Wang et al., 2012). Recently, recombinant protein subunit vaccine rVP4 and an inactivated vaccine were proven to be effective in preventing viral haemorrhagic disease caused by GCRV II in grass carps via intraperitoneal (i.p.) injection (Tian et al., 2013; Zeng et al., 2016). Therefore, the development of effective vaccines is critical for controlling pathogens.

Depending on the age and size of the fish, vaccines are commonly administered by i.p. or intramuscular (i.m.) injection, immersion or orally (Embregts & Forlenza, 2016). Protection is generally the highest with injection-vaccination, although it is also associated with stress for the fish and handling costs (Munang'andu & Evensen, n.d.). Similarly, the high costs of vaccine production restrain immersion vaccination. Therefore, the mass vaccination of fish exclusively via the oral route is the best compromise since it is needle free, has no size limitation, has a lower cost and is more convenient for farmers (Sommerset et al., 2005; Plant & LaPatra, 2011). Many reports suggest that non-encapsulated bacteria are suitable for oral vaccine vehicles, especially live commensals (Cui et al., 2015; Sun et al., 2010; Wilson & Hruby, 2005), since they retain their ability to colonise the intestine and persist long enough to deliver the antigen at mucosal surfaces (Guo et al., 2016). Recently, *Bacillus subtilis* spores were proved to be an ideal oral vaccine delivery system for presenting heterologous antigens to the gastrointestinal tract because of their advantages, such as non-pathogenicity, heat-stability and strong tolerances to the gastrointestinal environment (Wang et al.,

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2014; Tang et al., 2017).

The oral administration of antigens, in which intestinal uptake is expected, resulted in stimulation of innate and adaptive immune responses (Mutoloki et al., 2015; Munang'andu et al., 2015). Similar to systemic immune responses, antigen-presenting cells (APCs), such as monocytes and macrophages, in the lamina propria and intraepithelial B and T lymphocytes in the intestines of fish play an important role in antigen uptake (Zhang et al., 2010; Li et al., 2006; Fuglem et al., 2010; Rombout et al., 1985), which is linked to the expression of different cytokines (Kai et al., 2014). A cell-mediated immune response in fish mucosa is orchestrated by the CD4 and CD8 genes expressed in response to mucosal vaccination (Kai et al., 2014; Chettri et al., 2014; Martin et al., 2012; Overgard et al., 2013; Buonocore et al., 2012). In addition, the antibodies generated by mucosal vaccination are protective against microbial invasion into the intestinal mucosa (Kai et al., 2014; Ballesteros et al., 2013). Nevertheless, the challenge remains to demonstrate the mechanisms that mediate these processes in mucosal vaccination.

In this study, we constructed two recombinant spores expressing abundant fusion proteins of cotC-VP4 and cotC-NS38 on spores of wild-type *B. subtilis* GC5 isolated from grass carp. Through in vivo studies, grass carp specimens were orally administrated with recombinant *B. subtilis* spores, and then studies of the immunoprotective effects and immune response, including expression profiling of a suite of genes and antibodies, were undertaken. In addition, comparisons of the mechanisms of these two vaccines were performed.

2. Materials and methods

2.1. Fish

Grass carp approximately 8–10 cm in length (15–20 g) obtained from Wulonggang Aquatic Product Development Company (Guangdong Province, China) were maintained at Xiantao Fishery (Hubei Province, China) at approximately 28 °C and fed a commercial diet daily. Prior to the vaccination experiments, the fish were acclimatised for 2 weeks and then randomly sampled from the liver, kidney and spleen for the examination of the virus by RT-PCR to ensure the fish were naïve. Briefly, the total RNA of GCRV was extracted using TRIZOL Reagent (Invitrogen, USA). The total RNA (1 μg) were used for cDNA synthesis in a 25 μl reaction volume with M-MLV reverse transcriptase (Promega, USA). The synthesized cDNA was diluted with 75 μl of RNase-free water and used as template. According to the research, the primers for the detection of GCRV II targeting the *s6/s10* were designed, then PCR assay was performed. The primers are listed in Table 1.

2.2. Virus and in vivo viral titration

GCRV-HF was isolated from diseased grass carp (Hefei, Anhui Province, China) and purified according to the previous report (Pei et al., 2014). Briefly, gills and internal organs were collected and homogenized in NTE buffer (100 mM NaCl, 50 mM Tris-HCl, 10 mM EDTA, pH 7.2). The upper aqueous phase was collected after centrifugation at 8000*g* for 20 min, and the virus particles were concentrated by precipitation with 8% polyethylene glycol-6000 at 4 °C overnight. The pellet was dissolved in NTE buffer and centrifuged again at 8000 ×*g*. The supernatant was then ultracentrifuged at 30,000 rpm (Beckman Optima, SW41 rotor) for 90 min. The virus pellet was resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5). The virus was detected by a duplex PCR (Zeng et al., 2014), according to the research, the primers for the detection of GCRV-I and II targeting the S6 gene were designed, then PCR assay was performed. All PCR products were cloned into pMD18-T vector, then all the constructs were confirmed by complete sequencing (TSINGKE Biological Technology). Multiple sequence alignment was generated using ClustalW2, and then the virus was confirmed as belonging to GCRV II. Virus stock was titrated by in vivo infection experiments. Simply, the stock was diluted several times, then grass carp was injected intraperitoneally 100 μl the stock. Fish mortality was monitored daily for 14 days. Therefore, the amount used for challenge experiments was that required to achieve mortality above 90% in 14 days.

2.3. Bacterial strains, plasmids and transformation

The *B. subtilis* strain of GC5 from grass carp was routinely stored in our lab. The isolation and identification have been described previously (Guo et al., 2016). The total RNA of GCRV was extracted using TRIZOL Reagent (Invitrogen, USA). The total RNA (1 μg) were used for cDNA synthesis in a 25 μl reaction volume with M-MLV reverse transcriptase (Promega, USA). The synthesized cDNA was diluted with 75 μl of RNase-free water and used as template. The entire sequences of ORF of the GCRV-HF *s6* and *s10*, respectively, were amplified from the virus cDNA template using the specific primers GCRV-S6 F/R and GCRV-S10 F/R. To obtain an integration of the *cotc-s6* and *cotc-s10* fusion genes at *B. subtilis amy*E, a recombinant plasmid for double cross-over with the *B. subtilis* chromosome was constructed. We constructed the following elements: homologous arms *amy*E, resistance gene chloramphenicol (*Cm*), anchor protein *cotc*, including a promoter, linker $(GGGGS)_3$ and GCRV *s6/s10* in pMD-18T in turn, to generate recombinant plasmids named pAmy-CotC-S6 and pAmy-CotC-S10. Then, they were subsequently verified by complete sequencing. The recombinant plasmids were transformed into *B. subtilis* GC5 by the Spizizen method (Guo et al., 2016) with the brief modification of the addition of 4% Tween-20 in the preparation of competent cells. All the primers are listed in Table 1.

2.4. Culture, sporulation and identification of the recombinant spore

B. subtilis GC5-VP4 and GC5-NS38 were grown on chloramphenicol (Cm) resistance LB agar plates for 12–24 h. Chloramphenicol-resistant (Cm) clones were the result of a double cross-over recombination, leading to the interruption of non-essential amyE in the *B. subtilis* chromosome. Several Cm colonies were selected and grown on LB plates containing 0.5% starch overnight, and then the plates were stained with iodine to examine the amylase activity (Li et al., 2011).

GC5, GC5-VP4 and GC5-NS38 were grown in Difco Sporulation Medium (DSM) (Guo et al., 2016). Spores at 24 h were harvested and routinely treated for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Moreover, spore coat proteins were extracted from the spores using SDS-DTT extraction buffer (0.5% SDS, 0.1 M DTT, 0.1 M NaCl), then subjected to 12.0% SDS-PAGE and Western blotting to observe the expression of cotC-VP4/NS38 (Wang et al., 2014) using VP4/NS38-specific polyclonal antibodies (pAbs) and goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (HRP) (Thermo Scientific). Reactive bands were detected with Immobilon™ Western Chemiluminescent HRP Substrate (Millipore), and the pictures were taken by an Image Quant LAS 4000 system (GE Healthcare).

2.5. Surface display by immunofluorescence microscopy

The polyclonal antibodies against the S6 and S10 encoding proteins, VP4 and NS38, respectively, of GCRV-HF were produced by immunising rabbits according to conventional methods by using recombinant VP4 and NS38 expressed in *E. coli* (Tian et al., 2013). The IgG fraction of the rabbit antiserum was purified using a HiTrap Protein G column (GE Healthcare, USA). Thereafter, the specific pAbs were purified by affinity chromatography using NHS-activated Sepharose 4B (GE Healthcare, USA) coupled with recombinant VP4 or NS38 (Zhang et al., 2015).

To confirm the fusion expression of VP4 or NS38 protein on the surface of the spore of *B. subtilis* GC5, 1-ml sporulation cultures of GC5-VP4 and GC5-NS38 at 24 h were harvested, respectively. The samples were blocked with 10% BSA overnight ay 4 °C followed by incubation

Table 1
Primers used in this study.

Gene	Primer sequence[a][b]	Application
GCRV-S6	F: 5′-CTAGGATCCATGGGAAACGTCCAGACGAAC-3′ R: 5′-CCGCTCGAGCTAAGACGGAGGAGGCCAGTATC-3′	Plasmid construction
GCRV-S10	F: 5′-CGCGGATCCATGGCGGGTGTGTCTCTCAAC-3′ R: 5′-CGCTCGAGCTACAGCATCTGCGCGAATATCCGTCT-3′	Plasmid construction
GCRV-S8	F: 5′-ATGTATCTGGAACTGTTCATCGCTG-3′ R: 5′-TTATGGGCTCTTAGCCTTTGCCTTG-3′	Virus detection
amyE 5′	F: 5′ -TCACTAGCTAGCTCATTGCTCGGGCTGTATGACTG-3′ R: 5′-AAGCTTGACTGACCCGGGAATCTCACACCAT-3′	Plasmid construction
amyE 3′	F:5′-CCCGGGTCAGTCAAGCTTGAAAAGCCAAATAGGCGATC-3′ R: 5′-TCAAAAGTACTGTTACACCATCACTGTTCGTTCC -3′	Plasmid construction
CAT	F: 5′-ATTCCCGGGAGCACGCCATAGTGACTGGC-3′ R:5′-ATCCAAGCTTTCTAGAGGATCCCTCGAGGCGGCCGCGGTACCTTATAAAAGCCAGTCATTAGG-3′	Plasmid construction
CotC	F:5′-TTCTAGTCTAGATGTAGGATAAATCGTTTGGGC-3′ R:5′-CGGATCC*ACTACCGCCACCTCCACTACCGCCACCTCCACTACCGCCACCTCC*GTAGTGTTTTTTATGCTTTTTATAC-3′	Plasmid construction
CotC-S10	F:5′-TGTAGGATAAATCGTTTGGGC-3′ R: 5′-CTACAGCATCTGCGCGAATATCCGTCT-3′	PCR detection
amyE	F: 5′ -TCATTGCTCGGGCTGTATGACTG-3′ R: 5′-GTTACACCATCACTGTTCGTTCC -3′	PCR detection
β-actin	F: 5′-CCCAAAGCCAACAGGGAAAAGA-3′ R: 5′-GGCAGGGCATAACCCTCGTA-3′	Real-time PCR
MHC-I	F: 5′-CCTGGCAGAAAAATGGACAAG-3′ R: 5′-CCAACAACACCAATGACAATC-3′	Real-time PCR
MHC-II	F: 5′-GACTTCTACCCTCAACCAATCA-3′ R: 5′-CTGGCGTGCTCCACCATACA-3′	Real-time PCR
IgM	F: 5′-CATGATGACTGCACCAATGTTG-3′ R: 5′-TGAAATGTGCCTCCGATGCCT-3′	Real-time PCR
IgT1	F: 5′-CAATGTCTCAGTGTAAATCTTCC-3′ R: 5′-CATTTTGGTACGCTGCATCACA-3′	Real-time PCR
IgT2	F: 5′-TGTCTATATCATGTGGAAAGTG-3′ R: 5′-TTGAAACTTGTAATGGAGCGCTT-3′	Real-time PCR
IL-1β	F: 5′-TGTGACGCTGAGAGACGGAAA-3′ R: 5′-GAGTTTCAGTGACCTCCTTCAA-3′	Real-time PCR
IL-10	F: 5′-GTCATGCTTCTGCTTTCTGAAA-3′ R: 5′-TGGCATCCATAAGGACTATTGA-3′	Real-time PCR

[a] Restriction sites for plasmid construction are underlined.
[b] Linker $(GGGGS)_3$ are in italics.

with anti-VP4 IgG or anti-NS38 IgG at 37 °C for 2 h. FITC-labelled goat anti-rabbit IgG (Thermo, USA) was employed for visualisation. The wild-strain GC5 was used as a control. The images were acquired using a confocal microscope (Zeiss, Germany).

2.6. Vaccination and challenge

Immunisation trials were conducted on specific pathogen-free grass carp (100 fish per group).On 1, 2, 3, 21, 22 and 23 days, grass carps in the GC5-VP4 and GC5-NS38 groups were orally immunised with spores (2×10^9 spores/fish/d) with recombinant plasmids pAmy-CotC-S6 and pAmy-CotC-S10, respectively, while grass carps immunised with GC5 and PBS were used as a control. On 3, 7, 14 and 21 day post-vaccination (d.p.v.), the hindgut tissues were dissected for RNA extraction and stored in RNA*later* (Qiagen, USA) at −80 °C ready for qPCR. Furthermore, the fish were bled twice at weeks 3 and 6, respectively, as shown in Fig. 4*A. sera* were collected as described (LaFrentz et al., 2003) and then kept at −80 °C for ELISA.

On 42 d.p.v., 30 fish vaccinated with PBS, GC5, GC5-VP4 and GC5-NS38, respectively, were challenged by i.p. injection with GCRV-HF. Fish mortality was monitored daily for 14 days, and dead fish were examined by RT-PCR, using the primer GCRV-S8 F/R (Table 1), to confirm infection with GCRV. The relative percentage survival (RPS) was calculated by the following formula: RPS = [1-(% mortality of vaccinated fish/% mortality of control fish)] × 100.

2.7. Analysis of viable spores in intestinal tissues

After oral immunisation with 2×10^9 spores/fish, the hindgut tissues were recovered from sacrificed grass carp at 3, 6, 12, 24, 48 and 72 h and homogenized in PBS before plating serial dilutions on LB agar plates containing chloramphenicol (10 μg/ml). After that, several clones were picked up randomly and subjected to PCR amplification of *cotc-s10* using specific primers (Table 1). In addition, the adhesions of the spores to the intestinal mucus were examined by adhesive capacity analyses following the previous protocol (Guo et al., 2016).

2.8. RNA extraction and real-time qPCR

The total RNA of examined tissues was extracted using TRIZOL Reagent (Invitrogen, USA). First-strand cDNA was synthesized using the PrimeScript TM RT Reagent Kit with gDNA Eraser (Takara, Japan). The expression of the reference *β-actin*, a set of cytokine genes, as well as genes for cellular markers was detected by real-time qPCR performed in a DNA Engine Chromo 4 real-time system (BioRad, USA) with SsoAdvanced Universal SYBR Green Supermix (BioRad, USA). PCR conditions were as follows: 95 °C for 5 min and then 45 cycles of 95 °C for 20 s, 60 °C for 20 s and 72 °C for 20 s. The expression of genes was calculated as relative expression to *β-actin* using the $2^{-\Delta\Delta Ct}$ method, samples were analysed in triplicate and all data were reported as relative mRNA expression compared to the value of the PBS control group (Feng et al., 2011). All PCR products were cloned into pMD18-T vector, then all the constructs were confirmed by complete sequencing (TSINGKE Biological Technology). The primers for each gene are listed in Table 1.

2.9. Determining specific antibody of immunised grass carp by ELISA

The monoclonal antibodies (mAbs) against grass carp IgM were produced by immunising mice according to previous methods (Zhang

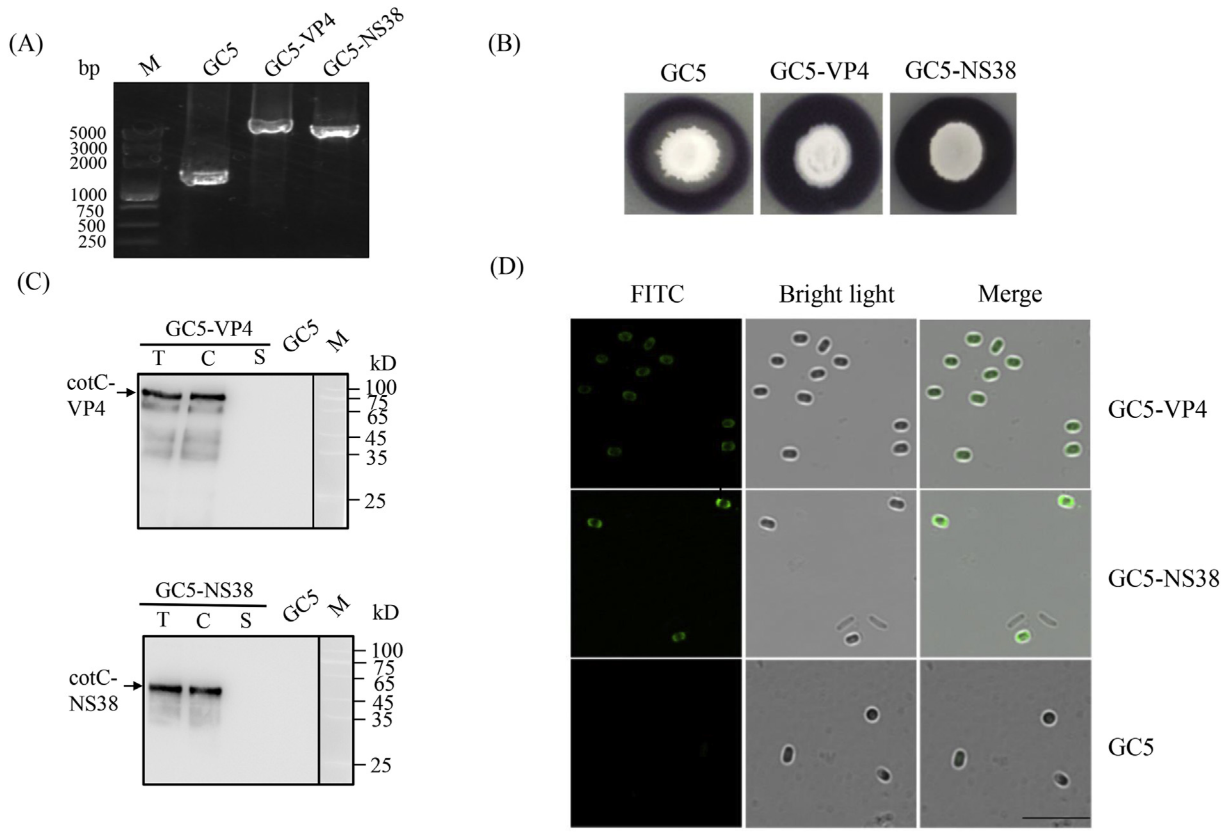

Fig. 1. Identification of the recombinant spore. (A) PCR analysis of *B. subtilis* genome. (B) Amylase activity analysis of recombinant *B. subtilis.* (C) Western blot analysis of cotC-VP4 or cotC-NS38 fusion protein expression on the recombinant spore coat, T: total cellular extract; C: spore coat proteins; S: sediment. (D) Expression analysis of cotC-VP4 or cotC-NS38 fusion protein by immunofluorescence. Immunofluorescent signals (green) are shown merged with the bright light of spores (scale bar: 10 μm). Magnification 63 × . (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

et al., 2010). Briefly, the recombinant CH3–4 domains of the grass carp IgM heavy chain were used to raise mAbs in BALB/c mice. Antibody specificity was determined by Western blot, ELISA and flow cytometry.

To test the specific IgM levels against VP4 or NS38 in the antisera of immunised fish, microplates were coated with 100 uL of recombinant VP4 or NS38 (10 μg/ml in carbonate-bicarbonate buffer) per well and incubated at 4 °C overnight. After blocking with 5% BSA in PBS for 1 h at 37 °C, serum samples were applied as a 1/100 dilution in PBS for 3 h at 37 °C. After washing with PBS three times, the plates were incubated with mouse anti-grass carp IgM (2 μg/ml in PBS-EDTA) for 1 h at 37 °C. Then, plates were incubated with HRP-conjugated goat anti-mouse IgG (Invitrogen, USA) diluted at 1:1000 for 1 h at 37 °C and next reacted with tetramethylbenzidine (TMB) substrate (Beyotime) for 10 min. Finally, the reaction was stopped by 2 M H_2SO_4, and the absorbance of each well was measured in a precision microplate reader (Biotek, USA) at 450 nm, and ELISA was performed in triplicate.

2.10. Statistical analysis

The statistic p values were calculated by one-way ANOVA with a Dunnett post-hoc test (SPSS Statistics, version 19, IBM). A p value of < 0.05 was considered significant. Differences were considered significant (shown as "*") at $p < .05$ and highly significant (shown as "**") at $p < .01$.

3. Results

3.1. Identification of the recombinant spore

After transformation of wild-type *B. subtilis* GC5, the fusion gene *cotc-s6* or *cotc-s10* was integrated into the *B. subtilis* chromosome at the *amyE* locus by double cross-over homologous recombination. Therefore, positive colonies with *cotC-S6* or *cotC-S10* integrated in the *B. subtilis* amyE locus were screened primarily by amylase activity analysis. As shown in Fig. 1B, a transparent zone was observed in wild-type *B. subtilis* GC5 when iodine was added in the starch plates owing to the expression of α-amylase from GC5 hydrolysing the starch in the plates. However, in plates with *B. subtilis* GC5-VP4 or GC5-NS38, a blue colour was produced by starch in the presence of free iodine since *amyE* was disrupted by the fusion of *cotc-s6* or *cotc-s10*, with no α-amylase secretion. The disruption of *amyE* was further confirmed by polymerase chain reaction (PCR) (Fig. 1A).

To confirm that VP4 or NS38 protein was expressed on the spore surface, antibodies against the VP4 or NS38 were used to perform western blot analysis. The result, Fig. 1C, showed that 78-kDa and 48-kDa bands were detected in the total cellular extract and spore coat protein extracts from recombinant spores GC5-VP4 and GC5-NS38, respectively. However, no similar band in the extracts from wild-type spores was detected, indicating the presence of the cotC-VP4 or cotC-NS38 fusion protein on the recombinant spore coat.

To validate the expression of VP4 or NS38 protein on the spore coat of recombinant *B. subtilis* GC5-VP4 or GC5-NS38, immunofluorescence analysis was performed. As shown in Fig. 1D, green fluorescence was detected on the spores of recombinant GC5-VP4 or GC5-NS38 after 24-h

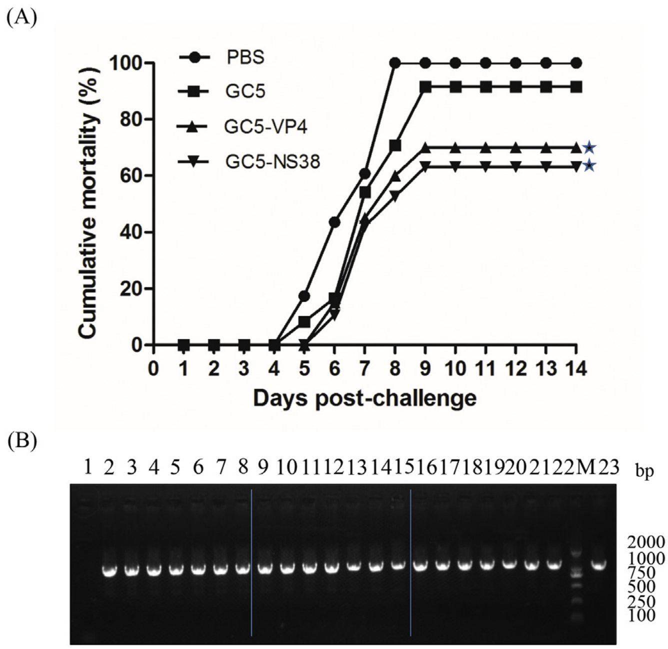

Fig. 2. (A) Cumulative mortality curves of vaccinated fish exposed to GCRV-HF. Mortality was monitored daily for 14 d post-challenge. *: the significant differences at the level of *$P < .05$ between immunised and control group of day 14. (B) PCR specific amplification the fragment of GCRV *s8* of moribund fish infected with GCRV. Lane 1: naive fish, Lane 2–8: PBS group, Lane 9–15: GC5-VP4 group, Lane 16–22: GC5-NS38 group, Lane M: 2000 bp DNA ladder marker, Lane 23: positive control (GCRV).

sporulation. By contrast, no green fluorescence was detectable in wild-type *B. subtilis* GC5, indicating that the observed fluorescence of recombinant GC5-VP4 or GC5-NS38 was specific.

3.2. Immunoprotection effect of vaccine

The vaccinated fish were challenged with GCRV-HF at 6-week p.v. and monitored for mortality for 14 days. As shown in Fig. 2A, mortality was first observed four days post-infection in the PBS/GCRV and GC5/GCRV groups, but one day later in the case of the GC5-VP4/GCRV and GC5-NS38/GCRV groups. Mortality reached its peak on the ninth day in most groups after the viral challenge. In addition, significantly lower cumulative mortality rates, 70% (70.00 ± 4.71%) in the GC5-VP4/GCRV group and 63.6% (63.56 ± 1.05%) in the GC5-NS38/GCRV group, were observed compared with those of the PBS/GCRV (100%) and GC5/GCRV (91.60 ± 7.07%) groups. Therefore, the protective efficacies of GC5-VP4 and GC5-NS38, in terms of RPS, respectively, were 30% (30.00 ± 4.71%) and 36.4% (36.44 ± 1.05%) with PBS as a control. The data represent means ± SD of two independent experiments. These indicated that recombinant *B. subtilis* GC5-VP4 and GC5-NS38 increased the survival rate of grass carp against GCRV, albeit with variations in the immunoprotective effect. The RT-PCR analysis of moribund fish revealed that GCRV was detected in the tissues of the diseased fish (Fig. 2B).

3.3. Colonisation of spores in intestinal tract

To evaluate the transit of GC5-NS38 spores in the grass carp intestine, we prepared spores and immunised via gavage at a dosage of 2×10^9 spores/fish. We also heat-treated them (80 °C, 20 min) to ensure the inactivation of all contaminating vegetative cells. At 3, 6, 12, 24, 48 and 72-h intervals thereafter, we collected hindguts to determine the count of spores present. Fig. 3A showed viable GC5-NS38 spores were present in the hindgut after 3 h at low levels, and reached maximum levels at 6 h. By 24 h, the GC5-NS38 spore count was still considerably high, and persisted steadily to 72 h. Besides, both wild-type and recombinant spores showed high adhesion rates (11.36–18.00%) to intestinal mucus (Fig. 3B), which may account for the considerable numbers of spores detectable at each time point, although spore counts gradually dropped over time.

3.4. Profile of gene expression in the intestine post-vaccination

Real-time qPCR was used to examine gene expression profiles in the intestine post-vaccination. The kinetics of pro-inflammatory cytokine IL-1β and anti-inflammatory cytokine IL-10 expression were evaluated and quantified. As shown in Fig. 4A and B, IL-1β transcripts were significantly up-regulated at 3 d.p.v. in GC5/GC5-VP4/GC5-NS38 groups compared with the PBS control group, whereas at 7 d.p.v. only the GC5-NS38 group was significantly up-regulated. The IL-10 transcript in the GC5-VP4 and GC5-NS38 groups was up-regulated compared with that in the control fish, albeit with variations in response times. With regard to antigen presentation, the mRNA of major histocompatibility complex class I (MHC-I), but not class II (MHC-II) molecules, was rapidly and significantly up-regulated in the GC5-NS38 group (Fig. 4C and D), whereas neither changed in the GC5 or GC5-VP4 group. Regarding IgM and IgT (Fig. 4 E and G), the IgM in the GC5-NS38 group and the IgT2 in the GC5-VP4 group were significantly up-regulated at 3 d.p.v., whereas that of IgT1 in the GC5-NS38 group occurred at 7 d.p.v. and in the GC5-VP4 group at 14 and 21 d.p.v. In addition, although IFN, Mx, CD4, CD8α, T-bet, and GATA3 transcription was also detected, the levels were not significantly different between the different experimental groups.

3.5. Specific antibody response of immunised grass carp

To test VP4 or NS38 protein-specific antibody response in the antisera of immunised grass carp, the fish were bled twice. Regarding the GC5-VP4 group, with the same antibody titer, the specific anti-VP4

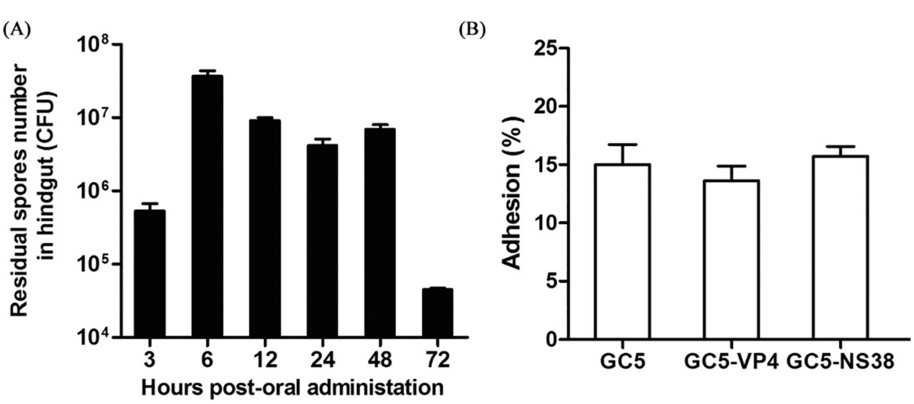

Fig. 3. Colonisation of spores in intestinal tract. (A) The spore counts obtained from hindgut tissues collected at the indicated time points after grass carp were immunised. The data represent means ± SEM of four fish. (B) Average adhesion percentages of the spores to intestinal mucus of grass carp. The data represent means ± SEM. The results are representative of the three independent experiments.

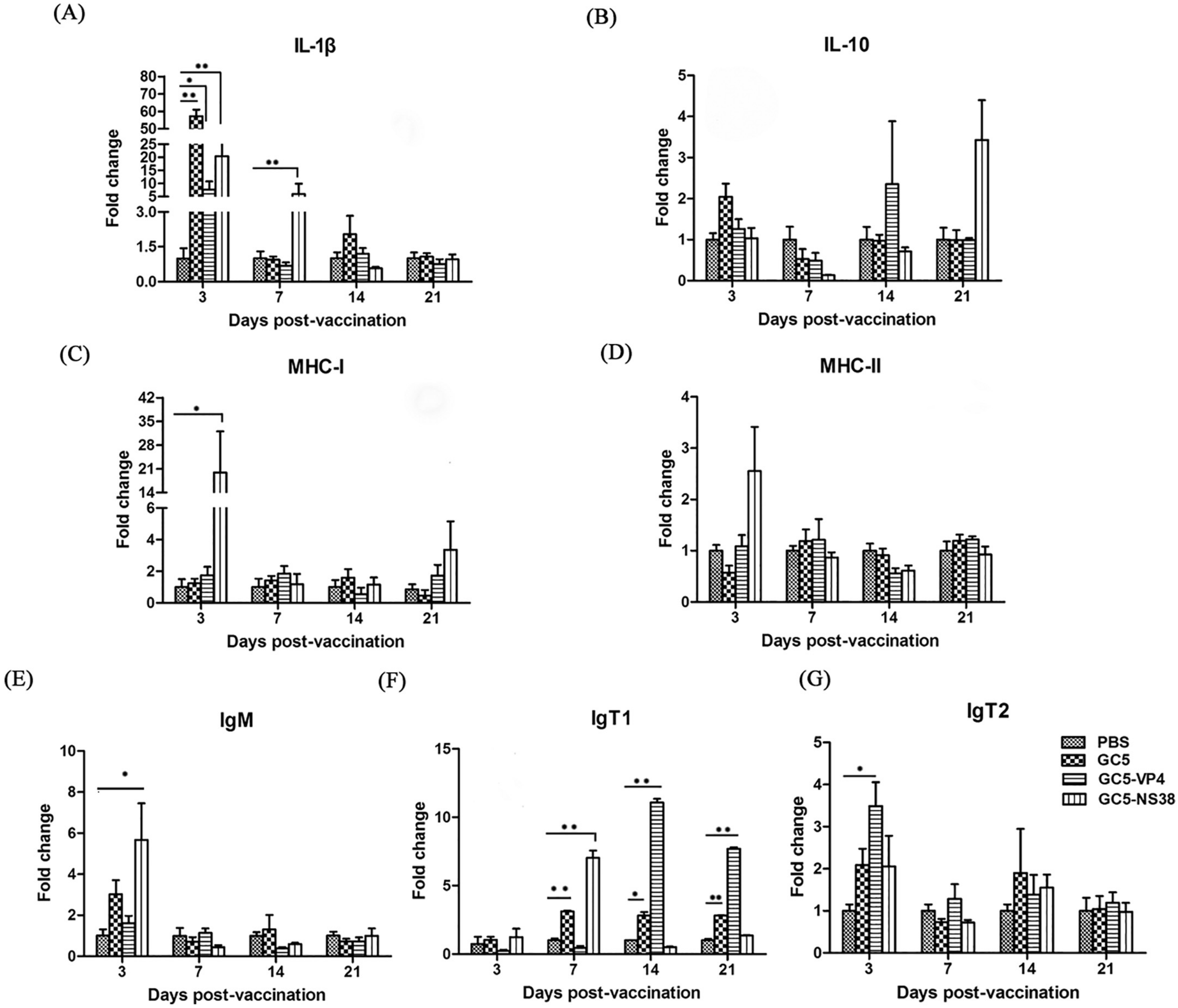

Fig. 4. Postvaccination qRT-PCR analysis of gene expression profiling in the intestine, including MHC-I, MHC-II, IL-1β, IL-10, IgT1, IgT2, and IgM. Expression of the indicated immune-relevant genes was normalized against the housekeeping gene *β-actin* and expressed relative to the PBS control group, which was used as 1-fold control. The error bar represents the standard deviation of mean ± SEM ($n = 4$). The statistical p values were calculated by one-way ANOVA with a Dunnett post-hoc test. Differences were considered significant (shown as "*") at $p < .05$ and highly significant (shown as "**") at $p < .01$.

levels had higher optical density values at 450-nm wavelength (OD_{450}) than those of the PBS group at 3 wk. after primary immunisation. At week 6 after the booster vaccination, the antibody level of the responder fish increased. In contrast, with regard to the GC5-NS38 group compared with the PBS group, although the specific anti-NS38 levels had slightly higher OD_{450} values, no significant difference was found between the primary and booster immunisations (Fig. 5).

4. Discussion

The aim of this study was to evaluate wild-type *B. subtilis* GC5 spores isolated from grass carp (Guo et al., 2016) as an oral vaccine delivery system to grass carp against GCRV. *B.subtilis* engineered to express heterologous antigens on the surface of the spore has been reported for oral or nasal delivery of antigens, conferring protective immunity in human and animal models (Wang et al., 2014; Tang et al., 2017; Lee et al., 2010). Several attributes of spores make them a particularly promising vaccine vehicle. First, they are non-pathogenic normally used as a probiotic for human and animal consumption (Barbosa et al., 2005; Liu et al., 2012). Second, as robust and dormant life forms, they are heat-stable and show strong tolerances to gastric (low pH) and intestinal (high bile concentration) conditions (Barbosa et al., 2005; Spinosa et al., 2000). Third, they can stimulate the intestinal immune system via the modulation of cytokine levels or competitive adherence (Plaza-Diaz et al., 2014). Thus, the current study applied *B. subtilis* GC5 as a delivery system to fish. The logic and novelty of our approach is that the wild-type *B. subtilis* GC5 from grass carp that we used might be able to adhere to the intestinal epithelial cells and mucus of grass carp, since adhesion to the mucus and epithelium are the foundation for probiotics to colonise intestinal mucosa and then play protective roles (Morelli, 2000).

With respect to GCRV (group II), although the hypothesis remains unproven, the S6 segment was predicted to encode the capsid proteins, whereas the S10 segment would encode the non-structural protein involved in the formation of viral inclusion bodies (Pei et al., 2014). The immunoprotective effect is related to the properties of antigens. In our

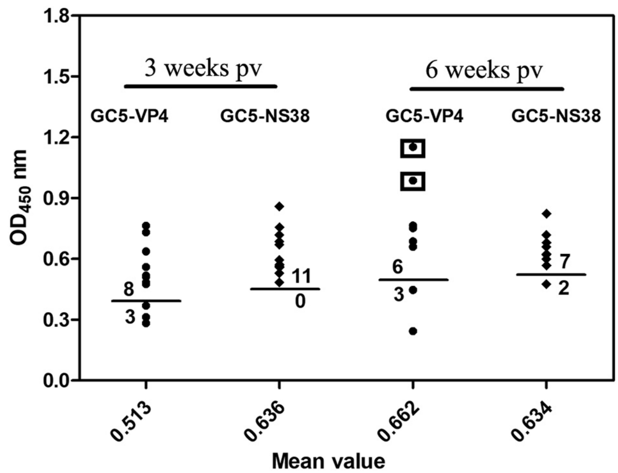

Fig. 5. Specific IgM antibody levels of grass carp immunised with GC5-VP4 or GC5-NS38. Specific anti-VP4 and anti-NS38 IgM levels of GC5-VP4 and GC5-NS38 post-vaccination, respectively. Each dot represents the OD_{450} value of ELISA for one fish, and the horizontal line in the graph signifies the average background OD_{450} value obtained from the control serum. The squares represent that the OD_{450} value obtained from the control serum of GC5-VP4 immunised serum was more than twice that of the control serum.

previous investigations, both DNA vaccine pC-S6 and pC-S10 induced an immunoprotective effect in grass carp against GCRV, albeit with variations (Chen et al., 2018) likely due to different ongoing responses. Likewise, in the current study, recombinant *B. subtilis* GC5-VP4 and GC5-NS38 increased the survival rate of grass carp against GCRV with variations in the immunoprotective effect. Above all, DNA vaccine pC-S6 and spore-based GC5-NS38 had the higher vaccine efficacy compared with pC-S10 and GC5-VP4, respectively. The effect of the same antigens was different because of vaccine formulation and inoculation routes and methods. Thus, we inferred that vaccine efficacy was not only related to antigen, but also vaccination strategies (Utke et al., 2008; Muhammad et al., 2012).

Before evaluating immune responses, we evaluated the survival of spores in the hindguts of grass carp. Using in vivo analysis, we found that a proportion of spores was still detectable 72 h after a single oral immunisation, which suggested that spores were not transient since they can adhere to the mucus and epithelium to colonise the intestinal mucosa. It was reported that the spore particles were taken up by M cells in the Peyer's patches in mammals because of the small size (1–2 μm) (Plaza-Diaz et al., 2014). Therefore, we speculated that the spores may be taken up in gut-associated lymphoid tissue (GALT) of grass carp. At 72 h, the significantly decreased numbers of spores may be related to germination and excretion.

Eliciting a complex and effective immune response would help to protect fish from viral infection (Embregts & Forlenza, 2016; Munang'andu et al., 2015). Indeed, many reports have indicated that the oral administration of antigens that were expected to be taken up by APCs such as macrophages, B cells, and T cells resulted in the up-regulation of genes related to the recruitment of immune cells (Rombout et al., 2011). In our study, the mRNA of MHC-I on the surface of APCs was rapidly and significantly up-regulated only in the GC5-NS38 group, suggesting that other cells were involved in antigen presentation in addition to professional APCs, since spores may be taken up by cells such as enterocytes in the gut (Chen et al., 2015). By contrast, no difference was found in the GC5-VP4 group. However, the up-regulation of IgM in the GC5-NS38 and GC5-VP4 groups occurred at 3 d.p.v. Since primary adaptive antibody immunity is commonly believed to occur at least after 14 d, the rapid rise of IgM and IgT1 mRNA indicated that these IgM^+ and $IgT1^+$ B cells might have participated in local antigen presentation or phagocytosis (Zhang et al., 2010). Taken together, these results that GC5-NS38 activated nonprofessional APCs besides IgM^+ B cells and GC5-VP4 activated $IgT1^+$ B cells, suggest that the presentation of GC5-VP4 and GC5-NS38 differed. In addition, the spores of GC-5 would not have had the same effect as spores expressing NS38 protein. This may relate to the fact that *B. subtilis* GC-5 was isolated from the intestinal tract of grass carp, which might have the intrinsic ability to be tolerated by the host.

Several probiotics can effectively modulate the expression of pro- and anti-inflammatory cytokines (Plaza-Diaz et al., 2014; Cerezuela et al., 2013). Similarly, IL-1β transcripts were significantly up-regulated at 3 d.p.v. in all groups except the PBS control, which showed that GC5 acted as a probiotic that activated the immune system. Additionally, mucosal antigen uptake is linked to the expression of different pro-inflammatory cytokines, such as IL-1β in the intestine (Kai et al., 2014; Chettri et al., 2014). Therefore, the upregulation of IL-1β in the GC5-NS38 group at 7 d.p.v. may be related to this. As a potent anti-inflammatory cytokine, IL-10 plays an essential role in preventing inflammatory and autoimmune pathologies. The IL-10 transcript in the GC5-VP4 and GC5-NS38 groups was up-regulated compared with that in the control group, suggesting that GC5-VP4 and GC5-NS38 could introduce a balanced pro-inflammatory/anti-inflammatory response. Since no significant difference was observed concerning IFN, Mx, CD4, CD8α, T-bet, and GATA3 transcription, the specific immunological mechanism is still unknown. Therefore, further work is needed to elucidate the details of these mechanisms.

The adaptive immune system, especially humoral, plays a crucial role in resistance to pathogen infection. The oral administration of antigens results in the stimulation of both systemic and mucosal antibody responses (Jiang et al., 2017). IgM plays a main role in the systemic immune response, whereas IgT is a relatively newly discovered intestinal mucosal immunoglobulin in fish (Zhang et al., 2010). Both immunoglobulins have been detected in the intestine (Chettri et al., 2014; Salinas et al., 2011; Hansen et al., 2005). Unlike IgM, IgT is specialised in mucosal surfaces, similar to IgA in mammals and accounts for the largest proportion of the B-cell population found in finfish intestines (Zhang et al., 2010). In our study, for mucosal immunity, IgT was detected by qPCR because of the lack of anti-IgT antibodies. The mRNA of IgT was significantly increased in the GC5-VP4 group at 14 and 21 d.p.v.; likewise, up-regulation occurred in the GC5-NS38 group at 7 d.p.v., suggesting that the local mucosal immune response was elicited by both GC5-VP4 and GC5-NS38, although the functional role of IgT has not been well understood/studied. With regard to IgM, up-regulation was not detected in the GC5-VP4 group or the GC5-NS38 group. Therefore, we speculated that the oral administration of recombinant spores elicited local (gut) IgT responses, but not IgM responses. For systemic antibody responses, significantly increased serum levels of specific IgM were observed in the GC5-VP4 group after booster immunisation, but not in the GC5-NS38 group. Taken together with the functional properties of the two proteins (VP4 protein = capsid proteins; NS38 protein = nonstructural proteins), we speculated that different antibody responses were activated by GC5-VP4 and GC5-NS38, but further studies are needed to understand their functions.

In summary, both the GCRV VP4 and NS38 proteins were efficiently displayed on the surface of grass carp-sourced *B. subtilis* GC5. Abundant colonisization spores were found in the hindguts of grass carp, which is conducive to the absorption and presentation of antigen. The oral administration of GC5-VP4 mainly elicited specific antibody responses, both systemic and mucosal, whereas GC5-NS38 mainly elicited an inflammatory response and the expression of MHC molecules on cells in the mucosa, but the mechanism is still unknown. GC5-VP4 and GC5-NS38 could increase the survival rate of grass carp against GCRV, suggesting that grass carp-sourced *B. subtilis* GC5 spores are a promising oral vaccine delivery system against GCRV.

Conflicts of interest statement

The authors have no conflicting commercial or financial interest in publishing this paper.

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